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14. ABSTRACT

We have found that the FGFR-4 Arg388 allele is strongly associated with the occurrence of prostate cancer and with metastasis and PSA recurrence in men undergoing radical prostatectomy. We will test the role of FGFR-4 in prostate cancer metastasis using autochthonous and orthotopic mouse models of cancer. We will also identify changes in gene expression due to the presence of the FGFR-4 Arg388 allele that lead to the metastatic phenotype. To this end we have established and carried out the initial characterization of trangenic mice with prostatic specific expression of FGFR-4 Arg388 and Gly388; constructed a FGFR-4 siRNA lentivirus and a PC3 siRNA cell line and identified Ehm2 as an FGFR-4 Arg388 regulated gene and characterized its role in prostate cancer. These key reagents will allow us to evaluate the effects of FGFR-4 on prostate cancer progression in vivo. Further studies of FGFR-4 Arg388 regulated gene expression in prostate cancer tissues are underway.

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INTRODUCTION

Fibroblast growth factors (FGFs) can control a multitude of cellular processes including proliferation, differentiation, survival, motility and angiogenesis. FGF2, FGF6, FGF8 and FGF17 are all expressed at increased levels in human prostate cancer and bind to FGF receptor-4 (FGFR-4) with high affinity. However, the role of FGFR-4 in human prostate cancer has not been systematically examined to date. Studies have shown that a germline polymorphism of the FGFR-4 gene resulting in expression of arginine at codon 388 (Arg^{388}) is associated with aggressive disease in patients with breast and colon cancers and sarcomas. We have found that homozygosity for the FGFR-4 Arg^{388} allele is strongly associated with the occurrence of prostate cancer in white men and the presence of the FGFR-4 Arg^{388} allele is also correlated with the occurrence of pelvic lymph node metastasis and PSA recurrence in men undergoing radical prostatectomy (1). Pooled cell lines expressing predominantly the FGFR-4 Arg^{388} or Gly^{388} allele were established by stable transfection in immortalized prostatic epithelial cells. Expression of FGFR-4 Arg^{388} resulted in increased cell motility and invasion through Matrigel when compared to cells expressing the FGFR-4 Gly^{388} allele. Microarray analysis has revealed that urokinase plasminogen activator system, matrix metalloproteases and cathepsin proteases may be involved the increased motility and increased metastasis seen in cells bearing the Arg^{388} allele. We hypothesize that FGFR-4, and in particular the FGFR-4 Arg^{388} variant, plays an important role in prostate cancer progression and metastasis based on correlative studies in humans. We propose to test this hypothesis in autochthonous and orthotopic mouse models of cancer. Furthermore, we hypothesize that these effects occur through specific changes in gene expression so we will carry out studies to identify changes in gene expression due to the presence of the FGFR-4 Arg^{388} allele that lead to the metastatic phenotype.

BODY

As outlined in our Statement of Work, we will seek to accomplish nine tasks in our three years of funding. Four of these tasks were to begin in Year 1. We have accomplished or made substantial progress on all four of these tasks, and have made progress on other tasks as well.

Task 1: Establish and characterize pathology in trangenic mice with strong prostate specific expression of FGFR-4 Arg^{388} and Gly^{388} variants (Months 1-18)

We have established FGFR-4 transgenic mice by injection of FGFR-4 Arg^{388} and Gly^{388} cDNAs under the control of the strong prostate specific ARR2Pb promoter into mouse blastocysts. We have performed quantitative RT-PCR on RNAs extracted from the prostates of these mice. As can be seen in Figure 1, expression of FGFR-4 is approximately 100-fold higher in the transgenic mice (note that graph is log scale).

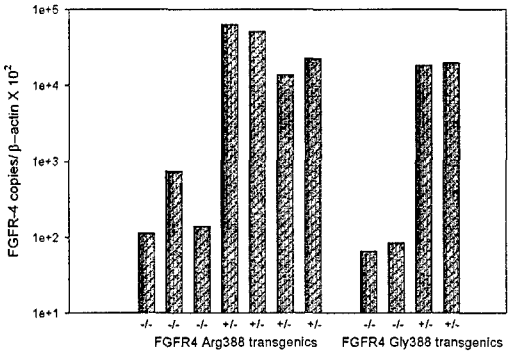

Figure 1. Expression of FGFR-4 in trangenic mice.

Expression of FGFR-4 was determined by quantitative RT-PCR in prostates from transgenic mice expressing FGFR-4 under the control of the prostate specific ARR2-Pb promoter. Note log scale.

After establishment of transgenic lines, we sacrificed mice at 2 month intervals and performed full necropsies to evaluate for any pathology, in particular, prostatic intraepithelial neoplasia or adenocarcinoma of the prostate. This work is mainly completed and we are analyzing the histopathology. Preliminary analysis reveals hyperplastic and dysplastic lesions in the ventral and dorsolateral prostate of transgenic mice but no invasive carcinoma has been identified. As expected, no pathology has been seen in other organs, since the ARR2Pb promoter is prostate specific.

Task 2: Cross FGFR-4 transgenics with TRAMP and ARR2Pb-myc mice and evaluate tumor progression (Months 12-36)

We will initiate crosses within the next month. We needed to wait to initiate these crosses until the initial characterization of the FGFR-4 Arg^{388} and Gly^{388} transgenic lines was completed since we were uncertain as to what the baseline pathology in the FGFR-4 transgenic mice would be.

Task 3: Collect tumors and establish cell lines from WT TRAMP and bitransgenic mice and evaluate effect of FGFR-4 Arg^{388} and Gly^{388} on proliferation, apoptosis and motility (Months 12-36)

Task 3 is dependent on Task 2 (see Task 2 above).

Task 4: Establishment and characterization of LNCaP and PC-3 cell lines with markedly decreased FGFR-4 expression by RNAi (Months 1-12)

We have constructed a lentivirus vector containing a SiRNA encoding sequence for FGFR-4. PC-3 prostate cancer cells have been infected and selection performed. By quantitative RT-PCR these cell have a 60% decrease in FGFR-4 mRNA (data not shown). By Western blot there is a marked decrease in FGFR-4 protein in the SiRNA expressing cells (Fig 2).

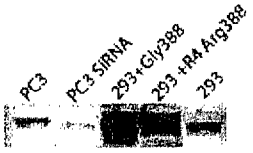

Figure 2. Western blot with anti-FGFR-4 antibody.

PC3: controls infected with empty virus; PC3 SiRNA: PC3 infected with SiRNA containing virus. 293 cells were transiently transfected with FGFR-4 Arg^{388} or Gly^{388} or empty vector and used for Western blot to demonstrate antibody specificity.

These cell lines are currently being characterized in terms of proliferation rate (doubling time), motility and invasion. LNCaP cell lines are currently under selection.

Task 5: Determination of the effect of decreased FGFR-4 expression in an orthotopic injection model of prostate cancer metastasis (Months 12-24)

We will assess the in vivo behavior of the cell lines established in Task 4, above, using an orthotopic injection model in nude mice in which cells are injected directly into the prostate. These experiments will begin shortly.

Task 6. Analysis of tumors from orthotopic injection model (Months 24-36)

This task is dependent on Task 5, above.

Task 7: Evaluation of expression of candidate proteins mediating the biological effects of FGFR-4 Arg^{388} and correlation with FGFR-4 genotype in white patients with prostate cancer (Months 1-18)

We have been assembling our tissue samples and should begin analysis of PAI-1, TIMP-1 and cystatin C expression shortly. It is far more cost effective to batch this analysis since the ELISAs need multiple wells for standards and controls. This approach also minimizes experimental variability.

Task 8: Identification of additional candidate genes mediating the biological effects of FGFR-4 Arg^{388} using targeted microarrays (Months 18-24)

We have identified a potential candidate gene that may mediate some of the biological effects of the FGFR-4 Arg^{388} polymorphic variant by analysis of expression microarrays. The Ehm2 gene is a member of the NF2/ERM/4.1 superfamily. Members of this superfamily all contain a FERM domain and are involved in membrane-cytoskeletal interactions. There is a strong correlation between Ehm2 gene expression and the metastatic potential of K-1735 and B16 melanoma cells (2). It is proposed that alterations of the expression level of Ehm2 are likely to be linked to one or more steps of cancer metastasis through regulation of interaction between cell surface transmembrane proteins and cytoskeletal proteins. Ehm2 is expressed at higher levels in PNT1A cells expressing FGFR-4 Arg^{388} when compared to cells expressing equivalent amounts of FGFR-4 Gly^{388} (Figure 3).

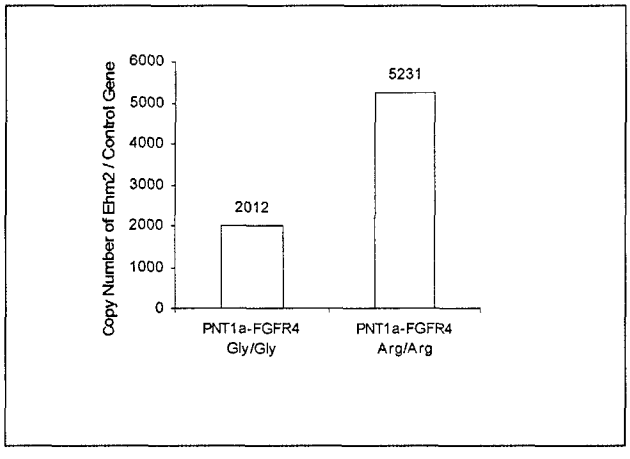

Figure 3. Increased expression of Ehm2 in PNT1A FGFR-4 Arg^{388} cells. Expression was quantitated using real-time RT-PCR of RNAs from the two cell lines. Data was normalized using β-actin transcript levels.

To analyze expression of Ehm2 at the protein level *in vivo* we performed immunohistochemical analysis of Ehm2 expression using a prostate cancer tissue microarray containing 32 matched normal and prostate cancer tissues. Staining was predominantly seen in benign and malignant epithelial cells (Figure 4) with some staining of smooth muscle. Stained slides were digitized and scored both for extent of staining (scale of 0-3) and intensity of staining (scale of 0-3). An average staining index was calculated from the extent of staining score for the three cores multiplied by the staining intensity score so that the staining index ranged from 0 (no staining) to 9 (extensive, strong staining). Normal prostate epithelium showed a staining index of 6.3 +/- 0.38 (mean +/- SEM) while the cancer tissues had a staining index 7.4 +/- 0.29. This difference was statistically significant ($p<0.05$, Mann Whitney). Overall, 27% of normal tissues showed low staining (index<6.0) while only 10% of cancers had low staining. In contrast, 40% of cancers showed maximal staining intensity (index =9) Vs 8% of normal epithelium. Of note was the finding that none of the cases with low Ehm2 staining

(staining index<6.0) had a PSA recurrence, while all cases with recurrence had moderate to strong Ehm2 staining (staining index 6-9). This difference is statistically significant (p=0.03, Fisher exact test).

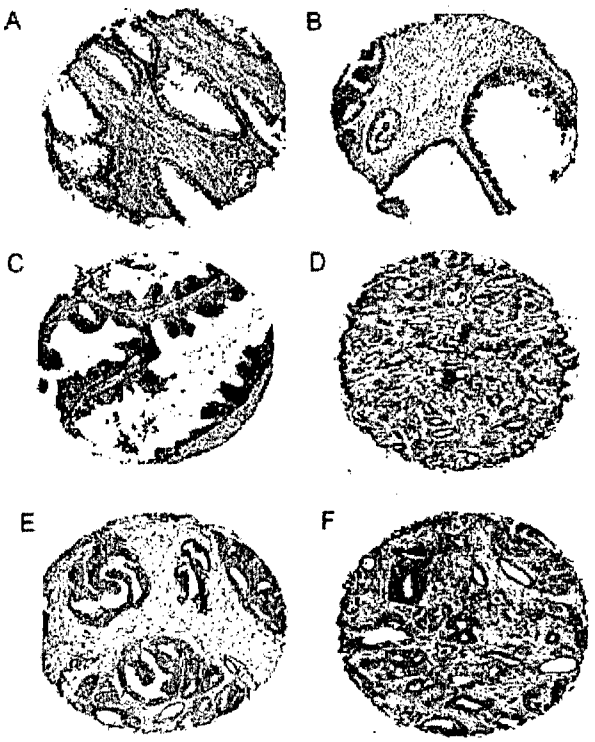

Figure 4. Expression of Ehm2 in normal prostate and prostate cancer. Immunohistochemistry with anti-Ehm2 antibody was performed using a tissue microarray of normal and prostate cancer tissues.
A. Normal prostate with weak staining of epithelium. B. Normal prostate with moderate epithelial staining. C. High grade prostatic intraepithelial neoplasia with strong staining. D. Prostate cancer with moderate staining. E, F. Prostate cancers with strong staining.

Ehm2 may regulate cytoskeleton interactions with transmembrane proteins and thus affect cancer cell adhesion. To evaluate the impact of Ehm2 on adhesion, we carried out two series of complementary experiments. PNT1a cells, which express low levels of Ehm2, were transiently transfected with an expression vector containing the Ehm2 cDNA. An example of one such experiment is shown in Figure 5A. Ehm2 expression was increased 12-fold in the transfected cells and this was associated with a 40% decrease in adhesion to collagen IV. This experiment was repeated 3 times with similar results. We then decreased Ehm2 mRNA in the LAPC4 cell line, which showed the second strongest Ehm2 expression among tested prostate cell lines, using SiRNA. As shown in Fig 5B, 24h after SiRNA transfection, Ehm2 expression showed an approximately 50% decrease in Ehm2 transcript level, while adhesion to collagen IV increased about 20% compared to control cells. This experiment was repeated 3 times with similar results each time (20, 17 and 18.5% increase). These results indicate that Ehm2 expression is associated with decreased adhesion to collagen IV by prostatic epithelial cells. Collagen IV adhesion has been previously shown to be inversely correlated with metastatic behavior in breast cancer (3).

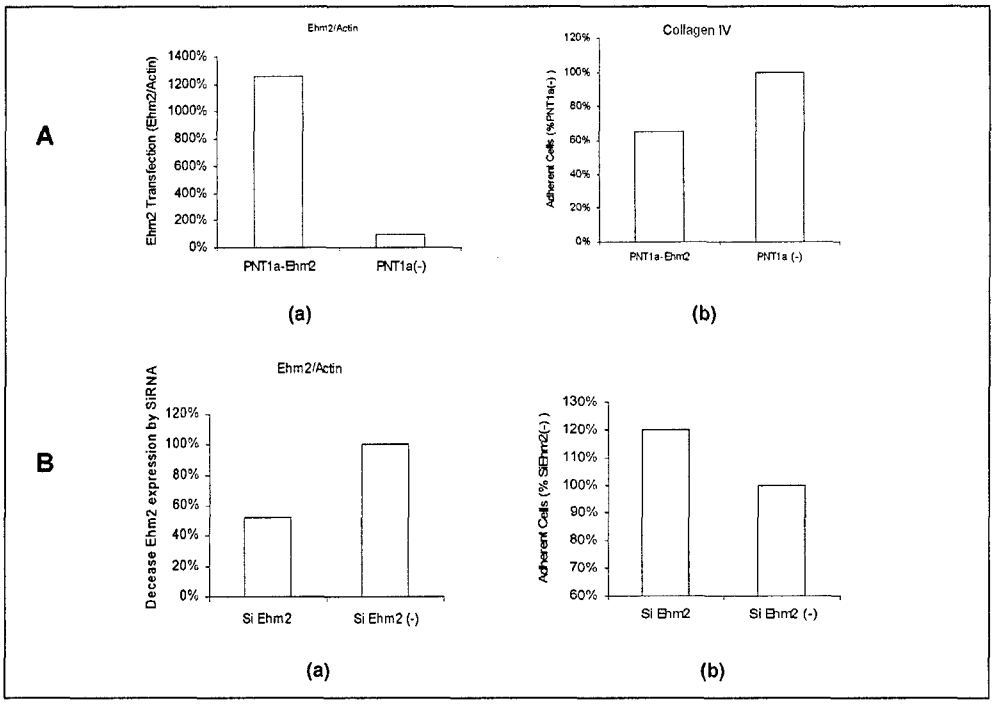

Figure 5. Ehm2 expression results in decreased collagen IV adhesion
A. PNT1A cells were transiently transfected with Ehm2 cloned into the pCMV-Tag2b expression vector or empty vector. (a) Expression of Ehm2 in transfectants by quantitative RT-PCR. (b) Collagen IV adhesion of Ehm2 and control PNTIA tranfected cells. One of 3 experiments with similar results is shown. B. LAPC4 prostate cancer cells were transiently transfected with Ehm2 or control siRNA. (a) Expression of Ehm2 in transfectants by quantitative RT-PCR. (b) Collagen IV adhesion of Ehm2 siRNA and control siRNA transfected LAPC4 cells. One of 3 experiments is shown, all of which had similar results.

In summary, we have identified Ehm2 as a gene that is upregulated by expression of the FGFR-4 Arg^{388} allele and can potentially promote metastasis. A manuscript describing our results is currently near completion and will be submitted shortly. We are also actively trying to identify and characterize additional FGFR-4 Arg^{388} regulated genes.

Task 9: Evaluation of expression of candidate proteins mediating the biological effects of FGFR-4 Arg^{388} and correlation with FGFR-4 genotype in white patients with prostate cancer (Months 24-36)
See Task 8 above. These experiments are planned for year three.

KEY RESEARCH ACCOMPLISHMENTS

* Establishment and initial characterization of FGFR-4 Arg^{388} and Gly^{388} trangenic mice.
* Construction of FGFR-4 siRNA lentivirus and establishment of PC3 siRNA cell lines
* Identification of Ehm2 as an FGFR-4 Arg^{388} regulated gene and characterization of its role in prostate cancer.

REPORTABLE OUTCOMES

* Establishment of FGFR-4 Arg^{388} and Gly^{388} trangenic mouse lines
* Establishment of PC3 FGFR-4 SiRNA cell line
* Manuscript describing role of Ehm2 in prostate cancer in preparation

CONCLUSION

Our published data indicates that FGFR-4 plays an important role in prostate cancer initiation and progression. We have developed key reagents that will allow us to assess the biological effects of FGFR-4 on prostate cancer progression *in vivo*. These *in vivo* experiments will be a major focus in the coming year. We have also identified Ehm2 as a metastasis promoting gene that is upregulated by expression of the FGFR-4 Arg^{388} variant. Further studies of this gene, as well as PAI-1, TIMP-1 and cystatin C in men with prostate cancer will yield new insights into the mechanism by which the FGFR-4 Arg^{388} can promote prostate cancer progression.

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